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Characterization of a bidirectional promoter shared between two human genes related to aging: *SIRT3* and *PSMD13*

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Abstract

The human *SIRT3* gene contains an intronic VNTR enhancer whose variability is correlated with life span. The *SIRT3* 5′ flanking region encompasses the *PSMD13* gene encoding the p40.5 regulator subunit of the 26S proteasome. Proteasome is a multicatalytic proteinase whose function declines with aging. *SIRT3* and *PSMD13* are linked in a head-to-head configuration (788-bp intergenic region). The molecular configuration of two genes that are both related to aging prompted us to search for shared regulatory mechanisms between them. Transfection experiments carried out in HeLa cells by deletion mutants of the *PSMD13–SIRT3* intergenic region showed a complex pathway of coregulation acting in both directions. Furthermore, linkage disequilibrium (LD) analyses carried out in a sample of 710 subjects (18–108 years of age) screened for A21631G (marker of *PSMD13*), and for G477T and $VNTR_{intron5}$ (markers of *SIRT3*), revealed high LD, with significantly different *PSMD13–SIRT3* haplotype pools between samples of centenarians and younger people.
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Keywords: *SIRT3*; *PSMD13*; Bidirectional promoter; Human longevity

Although aging is not regarded as an adaptive process, gene pathways affecting aging and life span are evolutionarily conserved [1,2]. Usually the conservation of aging-related paths is interpreted in terms of pleiotropic phenomena [3]; however, the significance of the evolutionary conservation of the paths is still under discussion [4,5]. In this frame, the evolutionarily conserved sirtuin 2 (*SIR2*) gene family is especially interesting. First, in model organisms the expression levels of *SIR2* modulate life span [6,7]; second, low-calorie diets that extend life span also promote sirtuin activity, showing that sirtuins may connect metabolism and aging [8]; third, Sir2 proteins exhibit nicotinamide dinucleotide phosphate (NAD^+)-dependent deacetylase activity that could account for the broad range of biological processes in which such proteins play a role, including gene expression, metabolism, and aging [9].

The *SIRT3* gene (11p15.5) is a human homologue of *SIR2* genes [10] that is expressed mainly in metabolically active tissues and is targeted to mitochondria through an N-terminal peptide sequence signal for mitochondrial localization [11–13]. The Ensembl database (http://www.ensembl.org) reports a list of putative orthologues of *SIRT3* (ENSG00000142082). For most of them a NAD-dependent deacetylase activity is reported in the database. Interestingly, it has been recently shown that murine Sirt3 activates mitochondrial functions and plays a crucial role in adaptive thermogenesis in brown adipocytes [14]. Taking into account the central role played by mitochondria [15] and lipid metabolism [16] in aging and longevity this finding suggests that in mammals *SIRT3* may play a role in the life span similar to that of its homologous *sir2* in yeast [6], the protozoan parasite *Leishmania* [17], worm [7], and fly [18]. According with this hypothesis, we found that *SIRT3* variability is associated with human longevity, likely through the enhancer activity of a VNTR (Variable Number of Tandem Repeats) occurring in intron 5 of the gene [19,20].

The *SIRT3* gene shows a head-to-head orientation (GenBank Accession No. NT_035113) with the proteasome 26S subunit non-ATPase 13 (*PSMD13*) gene, which encodes the p40.5 regulator subunit of the 26S proteasome. The 26S proteasome is

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a multicatalytic proteinase complex with a highly ordered structure composed of two complexes, a 20S core and a 19S regulator, working as natural machinery for the degradation of damaged proteins. Also for *PSMD13* (ENSG00000185627) the Ensembl database reports a list of putative orthologues that are involved chiefly in the degradation of ubiquitinated proteins, thus playing a role in cell cycle, cell cycle check point, and DNA replication, according to Reactome, a "knowledgebase" of biological pathways (http://www.reactome.org). The pivotal role played by proteasome in the degradation of abnormal proteins (for example oxidized proteins) links proteasome function to cellular senescence [21] and aging [22]. On the whole, we can infer that two genes that have a pivotal role in a number of cell pathways and lie head to head within a short chromosomal region are both related to aging and, possibly, to life span.

The unexpected configuration of *SIRT3* and *PSMD13* prompted us to investigate if the *PSMD13–SIRT3* intergenic region could act as a bidirectional promoter, capable of coordinating the expression patterns of the two genes. By transfection experiments we discovered not only that the region interposed between the two genes can regulate the transcription in both directions, but also that a common core rich in Sp1 sites plays a critical role in a shared regulatory mechanism. In addition, we investigated whether the gene/longevity association previously observed for *SIRT3* also involved *PSMD13*. The linkage disequilibrium studies presented here show that the haplotype pool defined by *PSMD13–SIRT3* variability differs in centenarians and in younger people. Our data provide the first evidence that two genes, both involved in aging and longevity, could share a common regulatory mechanism.

Results

Computer-assisted analyses of the PSMD13–SIRT3 intergenic region

Fig. 1 shows the two genes with their head-to-head orientation and the nucleotide sequence of the 788-bp intergenic region. Computer-assisted analyses revealed that the region has a high G+C content (67.19%) and contains two putative CpG islands (CpGPlot software); furthermore, the region lacks the typical TATA box sequence and its homologues (MatInspector software). As shown in Fig. 1B, the region contains multiple potential DNA motifs for AP-1, GATAs, NF-κB, and ZF5 and multiple binding sites for Sp1 factor clustered in proximity of the transcription start site of the *SIRT3* gene (+1 position in Fig. 1B, in which putative transcription factor binding sites are marked with arrows indicating the functional direction).

Molecular analyses of the PSMD13–SIRT3 intergenic region

By inspection of the *PSMD13–SIRT3* genomic region we observed that the *PSMD13* and *SIRT3* transcription start sites are located −72 and −32 nucleotides, respectively, from the translation initiation codons of the two genes. Therefore, the closeness of the transcription start sites suggested to us that the *PSMD13–SIRT3* intergenic region might act as a bidirectional promoter. Thus, to check our hypothesis and investigate the regulation of two divergently transcribed genes, we first verified the promoter activity of the entire intergenic region, then we searched for the core region that was essential for transcription in the direction of either *PSMD13* or *SIRT3*.

The PSMD13–SIRT3 intergenic region acts as a bidirectional promoter

We analyzed the promoter activity of the 788-bp intergenic region by using firefly luciferase as the reporter gene. This region was PCR-amplified from genomic DNA by using the primers SirtFor and SirtRev (see Fig. 1B). Then, the fragment was ligated in both directions into the promoterless pGL2-Basic vector upstream of the luciferase coding region. Transient transfection assays in HeLa cells revealed that the genomic fragment of 788 bp was sufficient for the expression of the firefly luciferase gene regardless of its orientation. Compared to the activity of the pGL2 vector alone (Fig. 2), the *PSMD13–SIRT3* intergenic region increased the luciferase activity by a factor of about 33 in the *SIRT3* direction ($p<0.0001$) and about 49 in the *PSMD13* direction ($p<0.0001$). On the whole, the transfection results showed that the regulation of the *SIRT3* and *PSMD13* gene expression could be coordinated through a bidirectional promoter.

Deletion analyses of the regulatory promoter

To check whether *SIRT3* and *PSMD13* genes share a common regulation pattern, we assembled partially deleted constructs and checked for their promoter activity in transient transfection experiments. In particular, we analyzed the functional effect of the multiple Sp1 sites located close to the transcription start site of *SIRT3* (Fig. 1B) in either the *SIRT3* or the *PSMD13* orientation. Deletion constructs were generated by cloning promoter PCR fragments in both directions upstream of the firefly luciferase gene into the pGL2-Basic vector. The reporter plasmids were transiently transfected into HeLa cells. The constructs, their promoter insert positions, and the cloning primer sequences are reported in Table 1. Promoter activity of the deletion fragments in either the *SIRT3* or the *PSMD13* direction was compared to that of the constructs containing the entire bidirectional promoter (pGL2/788/SIRT3 and pGL2/788/PSMD13 constructs). The results obtained by checking the entire group of constructs are shown in Fig. 3 and summarized in Table 2. In short, we see that Sp1 sites alone enhance the activity of the promoter in the *SIRT3* direction (A/SIRT3 construct) but not in the *PSMD13* direction (A/PSMD13 construct). In this orientation, ZF5/NF-κB/AP-1/GATA1-2 binding sites (D/PSMD13 construct) are required to restore the activity of the entire bidirectional promoter. However, in both directions, the absence of Sp1 sites (B/SIRT3 and B/PSMD13 constructs) causes a significant decrease in the promoter activity with respect to the entire intergenic region. On the whole, the results reported in Fig. 3 and summarized in

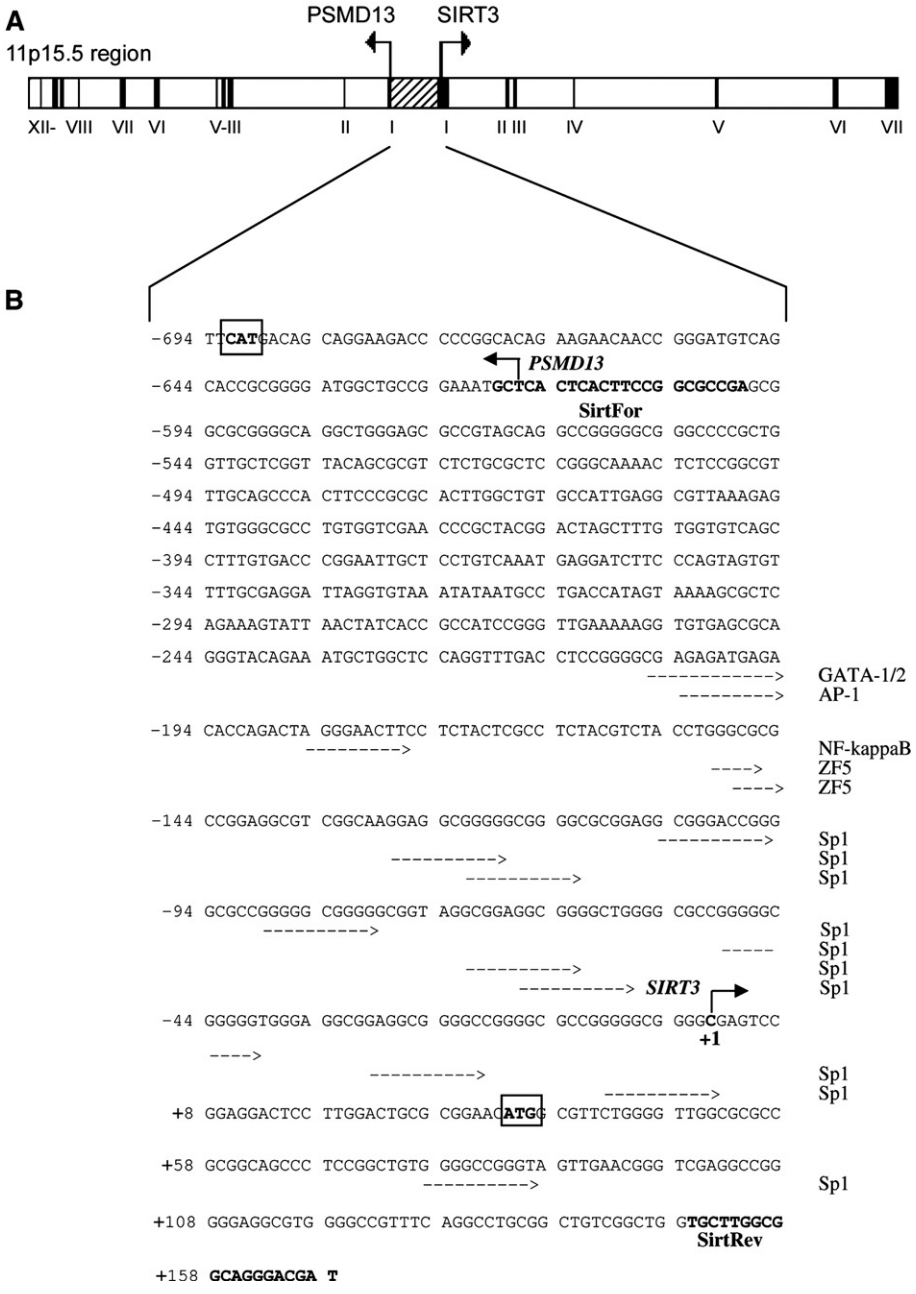

Fig. 1. (A) Structure of human *PSMD13* and *SIRT3* genes in the head-to-head orientation. The exons of the two genes are presented as filled boxes and indicated with roman numbers. The *PSMD13–SIRT3* intergenic region is presented as a hatched box. The bent arrows indicate the transcription direction. (B) Nucleotide sequence of the 788-bp intergenic region. Translation start codons of *SIRT3* and *PSMD13* genes are boxed. Transcription start sites are indicated by the bent arrows. The transcription start site of *SIRT3* is designated as "+1". Positive (negative) numbers are assigned to nucleotides downstream (upstream) of nucleotide +1. The analyzed putative transcription factor binding sites are shown with straight arrows. SirtFor and SirtRev primers used to clone the 788-bp intergenic region are in bold.

Table 2 show that the Sp1 sites play a critical role for the transcription in both directions, although synergies with further sites are required in the *PSMD13* orientation.

Linkage disequilibrium (LD) analysis

The finding that the *PSMD13* and *SIRT3* genes share a common regulation path prompted us to check whether the association we observed between longevity and *SIRT3* variability was extended to the *PSMD13* gene.

By using the A21631G marker of *PSMD13,* and the G477T and $VNTR_{intron5}$ markers of *SIRT3,* we carried out pairwise LD analyses in a sample of 710 unrelated subjects (see Materials and methods). We found statistically significant LD values (likelihood-ratio test) for all the pairs of markers ($A21631G_{PSMD13}$ and $VNTR_{SIRT3}$ $p=0.001$; $A21631G_{PSMD13}$ and $G477T_{SIRT3}$ $p=0.001$; $G477T_{SIRT3}$ and $VNTR_{SIRT3}$ $p=0.002$). Therefore, the linkage disequilibrium spans across the entire region depicted in Fig. 1A and involves the markers of both *PSMD13* and *SIRT3.*

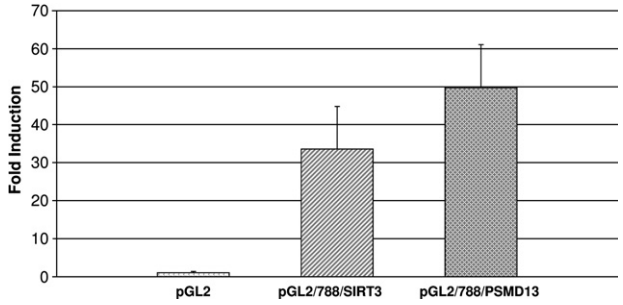

Fig. 2. Luciferase expression of pGL2/788/SIRT3 and pGL2/788/PSMD13 constructs reported as fold induction with respect to the pGL2-Basic vector. pGL2/788/SIRT3 and pGL2/788/PSMD13 contain the intergenic region in the *SIRT3* and *PSMD13* orientation, respectively. The values reported for transfection experiments are the means ± standard deviation of three independent duplicate experiments. The statistical significance of the differences between the promoter activity of the two constructs was tested by ANOVA and LSD post hoc tests.

To verify if the pool of *PSMD13–SIRT3* haplotypes changed in the population according to age, the sample was divided into two subsamples, the first made of 18- to 90-year-old individuals ($n=615$), the second made of 91- to 108-year-old individuals ($n=95$). The cut-off between age classes was chosen on the consideration that such a cut-off corresponds approximately to the surviving upper 0.1% of the population under study (Calabria, southern Italy). Table 3 shows the haplotype distribution in the two age groups. The exact test of population differentiation between the two subsamples revealed a significant difference between the haplotype pools ($p=0.019$). Therefore, as the population ages and survival selection operates, some haplotypes are preferentially lost from the haplotype pool (see for example the haplotype GG2 in Table 3), while others increase their frequency (see for example the haplotype AG4 in Table 3). On the whole, the haplotype analysis revealed that the chromosomal region associated with longevity comprises also the *PSMD13* gene.

Discussion

By exploring the neighborhood of the *SIRT3* gene we observed that the *PSMD13* gene lies very close to it (788 bp) in a head-to-head configuration. It was recently suggested that closely located bidirectional gene pairs whose transcription start sites are separated by less than 1 kb are common in the human genome and that such a bidirectional organization may control genes functionally related to each other [23–25]. Accordingly, the genes *PSMD13* and *SIRT3* could be functionally related. What is more, according to GenBank (http://www.ncbi.nlm.nih.gov/MapViewer) the head-to-head organization of the two genes is evolutionarily conserved in bird, rat, mouse, dog, chimpanzee, and human. Interestingly, the distance between the two genes increases throughout evolution (14 bp in *Gallus gallus,* 56 bp in *Rattus norvegicus,* 86 bp in *Mus musculus,* 157 bp in *Canis familiaris,* 555 bp in *Pan troglodytes,* where the regulatory sites display the same pattern as in humans, and 788 bp in *Homo sapiens*). This observation is intriguing, as it suggests an increasing complexity throughout evolution of a putative coregulation machinery shared between the two genes.

The aim of our work was: (a) to verify the bidirectional promoter activity of the *PSMD13–SIRT3* 788-bp intergenic region, (b) to identify a common regulation core in the promoter region, and (c) to verify whether the association with the longevity trait previously observed for *SIRT3* [19,20] involved *PSMD13*, too.

Bioinformatics analyses showed that the region is characterized by a GC content of 67% and comprises two CpG islands. These structural features are consistent with the findings that almost all the bidirectional promoters in mammalian genomes have a median GC content of 66%, and 77% of them are located in CpG islands [23,24,26]. The results of transfection experiments carried out by expression constructs containing the entire 788-bp intergenic region cloned in both *PSMD13* and *SIRT3* directions (Fig. 2) confirmed that such a region acts as a bidirectional promoter. Therefore we can give a positive answer to question (a).

As shown in Fig. 1B, the intergenic region contains a number of putative transcription factors binding sites such as AP-1, GATAs, NF-κB, ZF5, and Sp1. In particular, the GC boxes that bind Sp1 factors are clustered in proximity of the *SIRT3* transcription start site. A TATA box is absent. The lack of a TATA box is characteristic of the so-called housekeeping genes, but it was also observed in bidirectional promoters [26]. On the other hand, the presence of GC boxes close to the transcription start site is characteristic not only of TATA-less promoters, for which Sp1 factors are generally responsible for fixing the transcription start site, but also of bidirectional promoters [27,28]. By carrying out a series of transient transfection experiments with a set of deletion constructs (Table 1) cloned in both orientations, we discovered that Sp1 sites play a critical role in regulating gene expression in both directions (Fig. 3 and Table 2), although they act alone in the *SIRT3* orientation but in association with other transcription factors in the *PSMD13* orientation. In line with this observation, it has been documented that Sp1 factors may act in combination with other coactivator or corepressor factors to modulate transcription by physical or functional interaction [29,30]. On the whole, the entire set of results summarized in Table 2 suggests that *PSMD13* and *SIRT3*

Table 1
Deletion constructs, their promoter inserts, and cloning primers

Deletion construct	Promoter insert positions	Cloning primer sequences (5′→3′)
A/SIRT3	−145 to +169	ctcggtaccGCCGGAGGCGTCGGCAAG
A/PSMD13	−169 to +145	ctcggtaccATCGTCCCTGCCGCCAAGCA
B/SIRT3	−619 to −128	ctcggtaccGCTCACTCACTTCCGGCGCCGA
B/PSMD13	+128 to +619	ctcggtaccCTTGCCGACGCCTCCGGC
C/SIRT3	−174 to +169	ctcggtaccTCTACTCGCCTCTACGTC
C/PSMD13	−169 to +174	ctcggtaccATCGTCCCTGCCGCCAAGCA
D/SIRT3	−201 to +169	ctcggtaccGATGAGACACCAGACT
D/PSMD13	−169 to +201	ctcggtaccATCGTCCCTGCCGCCAAGCA

The 9-bp cloning adaptor is represented by lowercase characters. Promoter insert positions are related to the intergenic sequence in the *SIRT3* (A/SIRT3, B/SIRT3, C/SIRT3, D/SIRT3) and *PSMD13* (A/PSMD13, B/PSMD13, C/PSMD13, D/PSMD13) orientations.

are coregulated. Conclusive evidence of the role played by Sp1 and other regulatory factors (for example ZF5) in the co-regulation of the two genes will be provided by appropriate further experiments (for example gel-shift and mutagenesis experiments), which we are planning right now. In any case, on the basis of the results here presented, we can say that a fine

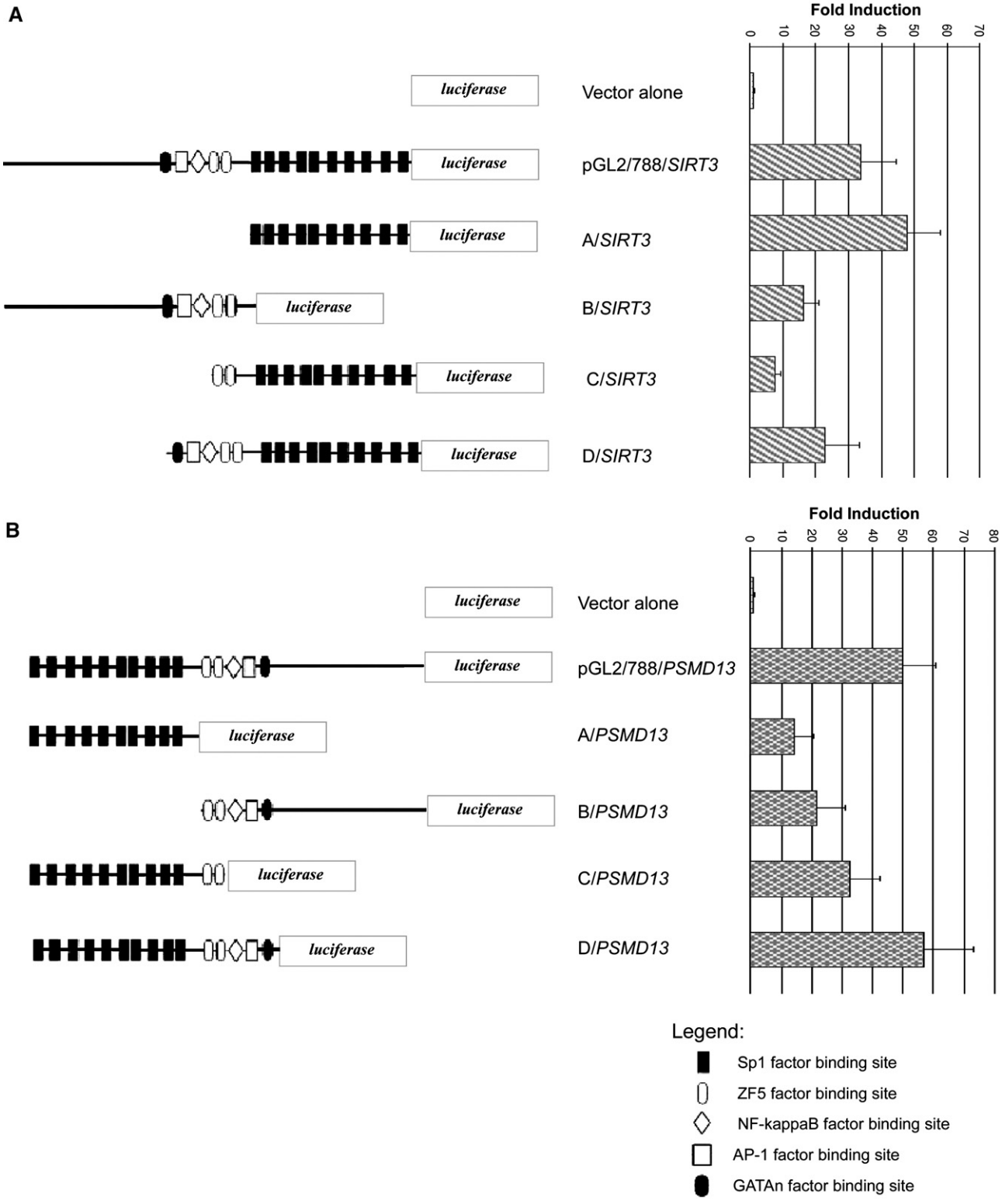

Fig. 3. Deletion analysis of the human *PSMD13–SIRT3* bidirectional transcription control region. On the left, the construct containing the whole 788-bp intergenic region and the constructs containing promoter fragments in the (A) *SIRT3* and (B) *PSMD13* orientation are shown with putative transcription factor binding sites. On the right, the activity of each construct is shown as fold induction with respect to the pGL2-Basic vector. The values reported for transfection experiments are the means ± standard deviation of three independent duplicate experiments.

Table 2
Synoptic table summarizing the results of the transfection experiments shown in Fig. 3

Deletion construct	Transcription regulatory elements	Transcription activity with respect to the entire promoter	*p* value
A/SIRT3	Multiple Sp1 binding sites only	Up-regulation	0.001
A/PSMD13		Down-regulation	0.000
B/SIRT3	Absence of multiple Sp1 binding sites	Down-regulation	0.000
B/PSMD13		Down-regulation	0.000
C/SIRT3	Multiple Sp1 binding sites plus two ZF5 binding sites	Down-regulation	0.001
C/PSMD13		Down-regulation	0.001
D/SIRT3	Multiple Sp1 binding sites plus two ZF5 binding sites and	Down-regulation	0.013
D/PSMD13	NF-κB/GATA1 binding sites	Same activity	0.156

The *p* values refer to the null hypothesis of no difference between the transcription activity of the entire 788-bp promoter and the transcription activity of the deletion construct (ANOVA and LSD post hoc tests).

game of coactivators and corepressors likely modulates a concerted expression of the two genes (question (b)).

Finally, through pairwise linkage disequilibrium analyses carried out between markers of *PSMD13* and *SIRT3* in a population sample including centenarians (Table 3), we showed that the entire chromosomal region encompassing the two genes is associated with the longevity phenotype (question (c)).

The observation that *PMSD13* and *SIRT3* share a bidirectional promoter (and that this architecture is conserved along evolution) is very interesting given that both genes are involved in aging. Usually, when more genes are clustered in the genome, their organization is selected relative to a possible complex phenotype to which such genes contribute. It would be surprising if this phenotype was aging, since several theories support the idea that aging is not a programmed and adaptive process, but a process due mainly to accumulation of stochastic mutations and that longevity is due, at most, to antagonist pleiotropic phenomena [3,4]. On the other hand, recent studies show that gene order in eukaryotic genomes is not completely random, but that genes with comparable and/or coordinated expression tend to be clustered together [31,32]. On the basis of this, the evidence for a common regulation between *SIRT3* and *PSMD13* genes is very intriguing considering that the two genes contribute to the same phenotype. However, it is still under discussion whether coexpressed genes have been linked together by natural selection to facilitate their expression or whether the genes are coexpressed simply because of their physical proximity in the genome. Several studies show that natural selection acts to preserve linked pairs of coexpressed genes [33,34]. Indeed, adjacent pairs of essential genes are preferentially conserved along evolution. The close proximity of the genes could be an adaptation that facilitates the coregulation of their transcription. It was observed that coexpressed genes remain linked more often than expected, which indicates that selection might favor their retention as a pair. It was also found that clusters of essential genes are in regions of low recombination and that larger clusters have lower recombination rates [35]. The selection could act to modify both the intragenomic variation in the recombination rate and the distribution of genes, thus determining the coevolution of gene order and recombination rate. A landmark in this field could be a recently published paper that reports results on head-to-head gene organization [36]. The authors carried out a systematic investigation of bidirectional gene pairs, focussing on structural features, evolutionary conservation, expression correlation, and functional association. The conclusion was that the head-to-head gene organization is ancient and conserved and may provide a fine mechanism of transcriptional coregulation based on gene organization. In particular, by comparing 42 head-to-head human gene pairs to their orthologue pairs in chicken and *Fugu,* the authors identified 10 pairs (20 genes) for which important conserved functions could be assumed: the *PSMD13–SIRT3* gene pair was one of them.

These considerations suggest an intriguing question: why did "Mother Nature" conserve a common pathway of regulation between two genes involved in a process that is believed to have come out of natural selection? It has been recently proposed that a programmed and altruistic aging may occur in higher eukaryotes [5]. Our findings are in line with this idea, although the deep evolutionary force that has driven such an architecture along evolution needs to be explored.

Table 3
PSMD13–SIRT3 haplotype pools in 18- to 90- and 91- to 108-year-old subjects

Haplotype	18- to 90-year-old subjects (n=615)		91- to 108-year-old subjects (n=95)	
	RF (%)	SE	RF (%)	SE
AG1	2.0	0.4	1.1	0.8
GG1	11.3	0.9	10.1	2.2
AT1	0.4	0.2	0.0	0.0
GT1	28.4	1.3	29.3	3.2
AG2	0.3	0.2	1.6	1.0
GG2	10.1	0.9	4.7	1.6
GT2	0.4	0.2	0.5	0.6
AG3	2.0	0.4	2.6	1.2
GG3	19.6	1.2	18.3	2.9
GT3	0.1	0.1	0.6	0.6
AG4	22.8	1.2	28.6	3.3
GG4	0.9	0.3	1.6	0.9
AT4	0.2	0.1	0.0	0.0
GT4	0.2	0.1	0.0	0.0
AG5	0.4	0.2	1.0	0.7
GG5	0.2	0.1	0.0	0.0
AG6	0.7	0.2	0.0	0.0

The markers used for haplotype analysis are the following (in order): A21631G for *PSMD13*, G477T and 1–6 $VNTR_{intron5}$ for *SIRT3*. Haplotype relative frequencies (RF) and standard errors (SE) are ×100.

Materials and methods

Molecular analyses of the PSMD13–SIRT3 intergenic region

Bioinformatic analyses

The GC content of the 788-bp common 5′ region shared by the *SIRT3* and *PSMD13* genes and detection of areas rich in CpG islands were performed using CpGPlot software (http://bioweb.pasteur.fr/seqanal/interfaces/cpgplot/html). Prediction of putative transcriptional factor binding sites was performed using MatInspector software (http://www.genomatix.de/matins).

Construction of reporter gene plasmids

The 788-bp *PSMD13–SIRT3* intergenic region was PCR amplified from human genomic DNA by using the forward primer SirtFor and the reverse primer SirtRev (Fig. 1B). The primers contained a 9-bp cloning adaptor with a *Kpn*I restriction site. The PCR was carried out in 100 μl of a mix consisting of 1× buffer, 100 mM dNTPs, 500 nM primers, 1.5 mM $MgCl_2$, and 5 U of DNAzyme (Finnzyme). The reaction consisted of 25 cycles, each cycle consisting of a denaturation step (94°C for 60 s), an annealing step (55°C for 60 s), and an extension step (72°C for 60 s). The first cycle was preceded by a denaturation step of 1 min at 94°C and the last one was followed by an extension step of 3 min at 72°C. The resulting 788-bp fragment was purified by agarose gel electrophoresis (Wizard SV Gel; Promega) and digested with *Kpn*I enzyme (Promega) as recommended by the manufacturer. After enzyme digestion, the fragment was inserted using T4 DNA ligase (Promega) in both orientations into the *Kpn*I site upstream of the firefly luciferase reporter gene in the pGL2-Basic vector (Promega), yielding two plasmids, pGL2/788/SIRT3 (intergenic region in *SIRT3* orientation) and pGL2/788/PSMD13 (intergenic region in *PSMD13* orientation). DNA was transformed into Top10 *Escherichia coli* cells by electroporation according to standard protocols. pGL2/788/SIRT3 and pGL2/788/PSMD13 constructs were prepared using the Wizard Plus SV Minipreps DNA purification system (Promega). The constructs were sequenced with the BigDye Terminator Cycle Sequencing Ready Reaction Kit (PE Applied Biosystems) to check the correct insert orientation and to confirm that the sequences matched the original genomic sequences without PCR-generated errors.

Deletion analyses

Deletion constructs were generated by PCR amplification of promoter fragments by using as template the reporter plasmids pGL2/788/SIRT3. The constructs, their promoter inserts, and the cloning primers are reported in Table 1. The primers contained a 9-bp cloning adaptor with a *Kpn*I restriction site. PCR was carried out as described above. After enzyme digestion, PCR products were purified by agarose gel electrophoresis and then inserted in both orientations into the *Kpn*I-linearized pGL2-Basic. All the plasmids were sequenced to check the correct insert orientation and to confirm the sequence.

Promoter analysis by luciferase assay

HeLa cells were cultured in Dulbecco's modified Eagle's medium (Invitrogen) containing 4.5 g/L glucose and 2 mM L-glutamine supplemented with 5% fetal bovine serum (Invitrogen) and 1% penicillin/streptomycin (Invitrogen). The cells were cultured in a water-humidified incubator at 37°C in 5% CO_2/95% air. HeLa cells (1×10^5) were transferred into 24-well plates with 500 μl of regular growth medium/well the day before transfection. Transfections were performed with the Fugene6 reagent as recommended by the manufacturer (Roche Molecular Biochemicals) with a mixture containing 1 μg of each reporter plasmid and 2 ng of pRL-CMV (Promega), a plasmid that contains the *Renilla* luciferase gene under the cytomegalovirus promoter and is utilized as an internal control to normalize the effects of transfection efficiencies. Cells were lysed 24 h after transfection by applying 50 μl Passive Lysis Buffer of the Dual Luciferase Reporter Assay Kit (Promega) into each well of the 24-well plate. Twenty microliters of cell lysate was used for luciferase reporter assay by using the same kit according to the manufacturer's protocol. Light intensity was quantified in a Lumat LB9507 luminometer (EG&G Berthold). The luciferase activity of the reporter plasmids was normalized to the *Renilla* luciferase activity. Each transfection experiment was carried out three times in duplicate. ANOVA and LSD post hoc tests were used to check the significance of the difference between the fold induction value of the deleted construct and that of the construct containing the entire 788-bp *PSMD13–SIRT3* intergenic region.

Population genetic analyses

Population sample

A population sample of 710 subjects was analyzed. All the subjects lived in Calabria (southern Italy) and their origin in the area had been ascertained up to the grandparents' generation (interview). The sample consisted of two subsamples: one included 18- to 90-year-old subjects (615 subjects, 262 males and 353 females), the other 91- to 108-year-old subjects (95 subjects, 31 males and 64 females). The younger group had been collected between 2000 and 2003 by an appropriate campaign addressed to Calabria University students and staff and to people who attended the University for the Elderly or used local thermal baths. The older group had been collected in the same period by consulting the Population Registers of the Municipalities of Calabria, contacting the potential probands by phone, and then visiting them in the case of positive answer to a first contact. After a detailed explanation of the aims of the genetic studies on aging carried out by our research group, the subjects who agreed to participate gave us a written informed consent and donated a blood sample for routine laboratory analyses and DNA preparation. People older than 60 years underwent a complete clinical and geriatric assessment. Subjects free of clinically overt pathologies and having blood and biochemical parameters in the normal age- and sex-specific range were enrolled in the study. In particular, the subjects in the older group belonged to the health categories A and B previously described [37].

DNA analyses

All the genotype data relevant to the G477T marker of *SIRT3* [14] and to the $VNTR_{intron5}$ marker of *SIRT3* [15] were already included in our database. Therefore DNA analyses were carried out only for genotyping *PSMD13* variability.

The A21631G marker located in exon 1 (position 21631) of the *PSMD13* gene (GenBank Accession No. AC136475) was analyzed by PCR and *Sac*I restriction analysis. A 465-bp fragment was amplified from genomic DNA (extracted from blood buffy coats) in 25 μl of reaction mixture containing 200 mM each dNTP, 0.5 μM both forward (5′-GACATCCCGGTTGTT-CTTCTG-3′, nt 21551, GenBank Accession No. AC136475,) and reverse (5′-CTACTCCTGAACCGTTTTAGT-3′, nt 22015, GenBank Accession No. AC136475,) primers, 1.5 mM $MgCl_2$, 1× polymerase buffer, 1 unit of EuroTaq DNA polymerase (EuroClone). The PCR consisted of 30 cycles, each cycle consisting of a denaturation step (95°C for 60 s), an annealing step (55°C for 60 s), and an extension step (72°C for 60 s). The first cycle was preceded by a denaturation step of 45 s at 95°C and the last one was followed by an extension step of 7 min at 72°C. A 20-μl amount of amplified DNA was digested by the restriction enzyme *Sac*I (5 U) for 3 h at 37°C as recommended by the manufacturer. The fragments were separated by electrophoresis on a 2.5% agarose gel and stained with ethidium bromide. The 465-bp PCR fragment contains two *Sac*I restriction sites, one of them is not polymorphic and produces two fragments of 341 and 124 bp. The *Sac*I polymorphic site is located within the 124-bp fragment. The presence of the G base in this site (GA*G*CTC) produces two fragments of 43 and 81 bp.

Haplotype analyses

Allele frequencies of single markers were computed by gene counting from the observed genotypes, and Fisher's exact test was applied to verify Hardy–Weinberg equilibrium [38]. Pairwise LD analyses were carried out on marker A21631G of *PSMD13* and markers G477T and $VNTR_{intron5}$ of *SIRT3* [19,20]. LD between pairs of markers was tested by a likelihood-ratio test. As for genotype data with unknown haplotype phase, an empirical distribution of haplotype frequencies obtained by a permutation procedure was utilized [39].

Population haplotype frequencies were estimated by maximum-likelihood estimation [38,40]. The null hypothesis of no difference between haplotype pools in the two age groups (18- to 90- and 91- to 108-year-old subjects) was checked by ad hoc exact test [41]. All the statistical analyses were carried out by means of Arlequin 2.0 software.

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References

[1] V.D. Longo, C.E. Finch, Evolutionary medicine: from dwarf model systems to healthy centenarians, Science 299 (2003) 1342–1346.
[2] L. Partridge, D. Gems, Mechanisms of ageing: public or private? Nat. Rev. Genet. 3 (2002) 165–175.
[3] A.M. Leroi, et al., What evidence is there for the existence of individual genes with antagonistic pleiotropic effects? Mech. Ageing Dev. 126 (2005) 421–429.
[4] S.N. Austad, Is aging programmed? Aging Cells 3 (2004) 249–251.
[5] V.D. Longo, J. Mitteldorf, V.P. Skulachev, Opinion: programmed and altruistic ageing, Nat. Rev. Genet. 6 (2005) 866–872.
[6] M. Kaeberlein, M. McVey, L. Guarente, The SIR2/3/4 complex and SIR2 alone promote longevity in *Saccharomyces cerevisiae* by two different mechanisms, Genes Dev. 13 (1999) 2570–2580.
[7] H.A. Tissenbaum, L. Guarente, Increased dosage of a sir-2 gene extends lifespan in *Caenorhabditis elegans*, Nature 410 (2001) 227–230.
[8] E.J. Masoro, Role of sirtuin proteins in life extension by caloric restriction, Mech. Ageing Dev. 125 (2004) 591–594.
[9] B.J. North, E. Verdin, Sirtuins: Sir2-related NAD-dependent protein deacetylases, Genome Biol. 5 (2004) 224.
[10] R.A. Frye, Characterization of five human cDNAs with homology to the yeast SIR2 gene: Sir2-like proteins (sirtuins) metabolize NAD and may have protein ADP-ribosyltransferase activity, Biochem. Biophys. Res. Commun. 260 (1999) 273–279.
[11] P. Onyango, I. Celic, J.M. McCaffery, J.D. Boeke, A.P. Feinberg, SIRT3, a human SIR2 homologue, is an NAD-dependent deacetylase localized to mitochondria, Proc. Natl. Acad. Sci. USA 99 (2002) 13653–13658.
[12] B. Schwer, B.J. North, R.A. Frye, M. Ott, E. Verdin, The human silent information regulator (Sir)2 homologue hSIRT3 is a mitochondrial nicotinamide adenine dinucleotide-dependent deacetylase, J. Cell. Biol. 158 (2002) 647–657.
[13] E. Michishita, J.Y. Park, J.M. Burneskis, J.C. Barrett, I. Horikawa, Evolutionarily conserved and nonconserved cellular localizations and functions of human SIRT proteins, Mol. Biol. Cell 16 (2005) 4623–4635.
[14] T. Shi, F. Wang, E. Stieren, Q. Tong, SIRT3, a mitochondrial sirtuin deacetylase, regulates mitochondrial function and thermogenesis in brown adipocytes, J. Biol. Chem. 280 (2005) 13560–13567.
[15] A. Santoro, et al., Mitochondrial DNA involvement in human longevity, Biochim. Biophys. Acta (2006) (Electronic publication ahead of print).
[16] G. Atzmon, et al., Lipoprotein genotype and conserved pathway for exceptional longevity in humans, PLoS Biol. 4 (2006) e113.
[17] B. Vergnes, et al., Targeted disruption of cytosolic SIR2 deacetylase discloses its essential role in Leishmania survival and proliferation, Gene 19 (2005) 85–96.
[18] B. Rogina, S.L. Helfand, Sir2 mediates longevity in the fly through a pathway related to calorie restriction, Proc. Natl. Acad. Sci. USA 101 (2004) 15998–16003.
[19] G. Rose, et al., Variability of the SIRT3 gene, human silent information regulator Sir2 homologue, and survivorship in the elderly, Exp. Gerontol. 38 (2003) 1065–1070.
[20] D. Bellizzi, et al., A novel VNTR enhancer within the SIRT3 gene, a human homologue of SIR2, is associated with survival at oldest ages, Genomics 85 (2005) 258–263.
[21] L. Farout, B. Friguet, Proteasome function in aging and oxidative stress: implications in protein maintenance failure, Antioxid. Redox Signaling 8 (2006) 205–216.
[22] N. Chondrogianni, E.S. Gonos, Proteasome dysfunction in mammalian aging: steps and factors involved, Exp. Gerontol. 40 (2005) 931–938.
[23] K.O. Koyanagi, M. Hagiwara, T. Itoh, T. Gojobori, T. Imanishi, Comparative genomics of bidirectional gene pairs and its implications for the evolution of a transcriptional regulation system, Gene 353 (2005) 169–176.
[24] N. Adachi, M.R. Lieber, Bidirectional gene organization: a common architectural feature of the human genome, Cell 109 (2002) 807–809.
[25] D. Takai, P.A. Jones, Origins of bidirectional promoters: computational analyses of intergenic distance in the human genome, Mol. Biol. Evol. 21 (2004) 463–467.
[26] N.D. Trinklein, et al., An abundance of bidirectional promoters in human genome, Genome Res. 14 (2004) 62–66.
[27] L. Lania, B. Majello, P. De Luca, Transcriptional regulation by the Sp family proteins, Int. J. Biochem. Cell Biol. 29 (1997) 1313–1323.
[28] G. Suske, The Sp-family of transcription factors, Gene 238 (1999) 291–300.
[29] K.D. Fischer, A. Haese, J. Nowock, Cooperation of GATA-1 and Sp1 can result in synergistic transcriptional activation or interference, J. Biol. Chem. 268 (1993) 23915–23923.
[30] H. Sakai, A. Jinawath, S. Yamaoka, Y. Yuasa, Upregulation of MUC6 mucin gene expression by NFκB and Sp factors, Biochem. Biophys. Res. Commun. 333 (2005) 1254–1260.
[31] L.D. Hurst, C. Pal, M.J. Lercher, The evolutionary dynamics of eukaryotic gene order, Nat. Rev. Genet. 5 (2004) 299–310.
[32] H. Caron, et al., The human transcriptome map: clustering of highly expressed genes in chromosomal domains, Science 291 (2001) 1289–1292.
[33] L.D. Hurst, E.J.B. Williams, C. Pal, Natural selection promotes the conservation of linkage of co-expressed genes, Trends Genet. 18 (2002) 604–606.
[34] G.A.C. Singer, A.T. Lloyd, L.B. Huminiecki, K.H. Wolfe, Clusters of co-expressed genes in mammalian genomes are conserved by natural selection, Mol. Biol. Evol. 22 (2004) 767–775.
[35] C. Pal, L.D. Hurst, Evidence for co-evolution of gene order and recombination rate, Nat. Genet. 33 (2003) 392–395.
[36] Y.Y. Li, et al., Systematic analysis of head-to-head gene organization: evolutionary conservation and potential biological relevance, PLoS Comput. Biol. 2 (2006) e74.
[37] C. Franceschi, et al., Do men and women follow different trajectories to reach extreme longevity? Italian Multicenter Study on Centenarians (IMUSCE), Aging Clin. Exp. Res. 12 (2000) 77–84.
[38] B.S. Weir, Genetic Data Analysis, II, Methods for Discrete Population Genetic Data, Sinauer, Sunderland, MA, 1996.
[39] M. Slatkin, L. Excoffier, Testing for linkage disequilibrium in genotypic data using the expectation–maximization algorithm, Heredity 76 (1996) 377–383.
[40] L. Excoffier, M. Slatkin, Maximum-likelihood estimation of molecular haplotype frequencies in a diploid population, Mol. Biol. Evol. 12 (1995) 921–927.
[41] F. Rousset, M. Raymond, Testing heterozygote excess and deficiency, Genetics 140 (1995) 1413–1419.